COMPARATIVE PHENOMICS AND GENOMICS OF Carbapenem-resistant *Escherichia coli* FROM HUMAN AND BROILER CHICKEN

ERKIHUN AKLILU WOLDEGIORGIS

UNIVERSITI SAINS MALAYSIA

2023

COMPARATIVE PHENOMICS AND GENOMICS OF Carbapenem-resistant *Escherichia coli* FROM HUMAN AND BROILER CHICKEN

by

ERKIHUN AKLILU WOLDEGIORGIS

Thesis submitted in fulfilment of the requirements

for the degree of

Doctor of Philosophy

July 2023

ACKNOWLEDGEMENT

I would like to express my gratitude to God almighty for the gift of life and its blessings. I thank my main supervisor, Assoc. Prof. Dr. Azian Harun for her unreserved guidance and support. I would also like to thank my co-supervisor, Assoc. Prof. Dr. Kirnpal Kaur Banga Singh for her assistance. My heartfelt appreciation goes to Universiti Malaysia Kelantan (UMK) and the Faculty of Veterinary Medicine for allowing me to complete my studies. I want to thank C.W. Salma C.W. Zalati, Nani Izreen Mohd Sani, and Norsyamimi Farhana Mat Kamir, respectively, at Molecular Biology, Virology, and Bacteriology, and Laboratories of the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan and Yasmin Khairani Mohd Smadi from Universiti Sains Malaysia for their assistance in facilitating the laboratory works for me. I also wish to convey my heartfelt gratitude to my family members, Negalign, Muluselam, Giorgis, and Dinkayehu, for their unconditional moral and spiritual support. Especial thanks to brother Siyum Mekuanint Ayalneh (Zekarias), whose selfless spiritual assistance has been instrumental in helping my family and me in recent years, without which this work could not have been completed. I would like to dedicate this work to my three beautiful, intelligent, and loving daughters, Yohanna Anjali, Maryann, and Evana Erkihun. They are my inspirations, and their presence and love taught me patience and perseverance in the face of apparently insurmountable challenges.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS/ABBREVIATIONS

Symbols/Abbreviations	Definition
%	percentage
<	less than
>	more than
^{o}C	degree Celsius
β-	Beta
g	gram
h	hour
ml	milliliter
pmol	picomole
pH	potential of hydrogen
s	seconds
µg	microgram
µl	microliter
µg/ml	microgram/ milliliter
Zn^{2+}	Zinc ion
µm	micromolar
ABC	ATP-binding cassette
AcrA	Acriflavine resistance A
AcrB	Acriflavine resistance B
adk	Adenylate kinase

aer	Aaerobactin
afa	Afimbrial adhesins
AIM-1	Adelaide Imipenemase-1
AmpC	Ampicillin and carbenicillin
AMR	Antimicrobial resistance
ANI	Average Nucleotide Identity
ARGs	Antimicrobial resistance genes
arpA	Ankyrin-like regulatory protein
ast	Arginine succinyltransferase
ATCC	American Type Culture Collection
BacWGSTdb	Bacterial whole-genome sequence typing database
BHIA	Brain Heart Infusion agar
BIC-1	Biceˆtre carbapenemase
bla genes	Beta-lactamases genes
BLAST	Basic Local Alignment Search Tool
BRIG	BLAST Ring Image Generator
BSIs -	bloodstream infections
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
CA	Clavulanate
CC	Clonal complex
CDC	Center for Disease Control
CDS	Coding sequences
cgMLST	Core genome multilocus sequence typing

chuA,	*Escherichia coli* haem-utilization gene
CIM	Carbapenem inactivation method
CLSI	Clinical and Laboratory Standards Institute
cnf1	Cytotoxic necrotizing factor 1
CP-CRE	Carbapenemase-producing CRE
CPEC	Carbapenemase-producing *E. coli*
CPE	Carbapenemase-producing Enterobacterales
CRAB	Carbapenem resistant *A. baumannii*
CRE	Carbapenem resistant Enterobacterales
CRE-BSI	CRE blood stream infection
CREC	Carbapenem resistant *Escerichia coli*
CRKP	Carbapenem-resistant *Klebsiella pneumoniae*
CRPA	Carbapenem resistant *Pseudomonas aeruginosa*
CTX-M	Cefotaxime hydrolyzing β-lactamase-M
cva/cvi	Colicin V structural gene/ colicin V immunity gene
DAEC	Diffusely adherent *E. coli*
DDD	Defined daily doses
DDST	Double disk synergy test
DEC	Diarrhoeagenic *E. coli*
DHFR	Dihydrofolate reductase
DHP-I	Dehydropeptidase
DHPS	Dihydropteroate synthase
DIM-1	Dutch imipenemase

DOR	Doripenem
E. coli	*Escherichia coli*
eae	Intimin production gene
EAEC	Enteroaggregative *E. coli*
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enter invasive *E. coli*
EMB-	Eosin Methylene Blue
EPEC	Enteropathogenic *E. coli*
ESBL-EC	Extended-spectrum -lactamase (ESBL)-producing *E. coli*
ESBLs	Extended spectrum-lactamases
E-test-	Epsilometer test
ETP	Ertapenem
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal pathogenic *E. coli*
fimA	Type 1 fimbriae
fimC	Type 1 fimbriae (D-mannose-specific adhesin)
fumC	Fumarate hydratase
GES	Guiana extended spectrum
GIM	German imipenemase
GlcNAc	N-acetylglucosamine

gyrB	DNA gyrase
HAIs	Hospital-acquired infections
HGT	Horizontal gene transfer
hly	Hemolysins gene
HUS	Hemolytic uraemic syndrome
HUSM	Hospital Universiti Sains Malaysia
hylA	Haemolysin A
icd	Isocitrate/isopropyl malate
IMI-1	Imipenem-hydrolyzing beta-lactamase
IMP	Imipenem/Imipenemase
irp2	Iron regulatory protein
iss	Increased serum survival
iutA	aerobactin siderophore receptor
KPC	Klebsiella pneumoniae carbapenemase
LMICs	low- and middle-income countries
LPS	lipopolysaccharides
MAC	MacConkey
MALDI-TOF MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MATE	Multidrug and toxic compound extrusion
MBL	Metallo-beta-lactamases
mCIM	Modified CIM
mdh	Malate dehydrogenase

MDR	Multi-drug resistant
MEM	Meropenem
MFS	Major facilitator superfamily
mg/L	Milligram per liter
MGEs	mobile genetic elements
MHA	Mueller-Hinton agar
MHT	Modified Hodge test
MIC	Minimum inhibitory concentration
min	minute
MLST	Multilocus sequence typing
MNEC	Meningitis-associated *E. coli*
mprF	Multiple peptide resistance factor
MRSA	Methicillin-resistant *Staphylococcus aureus*
MurNAcN	Acetylmuramic acid
MVAG	Malaysian Veterinary Antimicrobials Guidelines
MyAP-AMR	Malaysian Action Plan on Antimicrobial Resistance
NA	Nutrient agar
NBM	New born meningitis
NDARO	National Database of Antibiotic-Resistant Organisms
NDM	New Delhi metallo-beta-lactamase
NFW	Nuclease free water
NGS	Next generation sequencing
NmcA	Not metalloenzyme carbapenemase A

NPET	Nascent peptide exit tunnel
NSAR	National Surveillance of Antimicrobial Resistance
OMPs	Outer membrane proteins
ORFs	Open reading frames
OXA	Oxacillinases
PABA	Para-aminobenzoic acid
PACE	Proteobacterial antimicrobial compound efflux
PAIs	Pathogenicity islands
papC	Pilus associated with pyelonephritis
PATRIC	Pathosystems Resource Integration Center
PBP2a	Penicillin-binding protein 2a
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
pho	*Phosphate regulating gene*
PTC	*Peptidyl* transferase center
QC	Quality control
RNA	Ribonucleic acid
RND	resistance-nodulation-division
rRNA	Ribosomal RNA
sat	Secreted autotransporter toxin
SEPEC	Sepsis-associated *E. coli*
sfa	S fimbriae
SFC-1	*Serratia fonticola* carbapenemase-1

SIM	Seoul imipenemase
SME	Serratia marcescens enzyme
SMR	small multidrug resistance
SNPs	single-nucleotide polymorphisms
spp.	Species
ST	Sequence type
STEC	Shiga toxin-producing *E. coli*
stx_1	Shiga toxin 1
stx_2	Shiga toxin 2
TAT	Turn-around time
TBE	Tris Borate EDTA buffer
tet	Tetracycline resistance gene
tetX gene	flavin-dependent monooxygenase gene (tetracyclin resistance)
TMP	Sulphonamide and Trimethoprim
TolC	Outer membrane protein required for hemolysin secretion in *E. coli*
tRNA	Transfer RNA
TSB-	Tryptone Soy Broth
tsh	Temperature-sensitive haemagglutinin
TZB	Tazobactam
UPEC	Uropathogenic *E. coli*
UPGMA	Unweighted pair group method with arithmetic mean

UTIs	Urinary tract infections
VIM	Verona integron-encoded metallo-beta-lactamase
VRE	Vancomycin-resistant enterococci
WGS	Whole genome sequencing
WHO	World Health Organization
WOAH	World Organization for Animal Health
XDR	Extensive drug-resistant

LIST OF APPENDICES

PERBANDINGAN FENOMIK DAN GENOMIK *Escherichia coli* RINTANG KARBAPENEM YANG DIISOLAT DARIPADA MANUSIA DAN AYAM

ABSTRAK

Kemunculan Enterobacterales (CRE) rintang karbapenem adalah sangat membimbangkan dan kawalan penyebaran strain ini merupakan salah satu keutamaan yang ditetapkan oleh Pertubuhan Kesihatan Sedunia (WHO). Di Malaysia, laporan terkini menunjukkan peningkatan kes-kes CRE yang dilaporkan di hospital am dan hospital tertiari. Walau bagaimanapun; laporan kes CRE pada haiwan, terutamanya haiwan penghasil makanan seperti ayam dan itik di Malaysia adalah pada tahap minimum. Selain itu, sehingga kini tiada kajian yang melaporkan perbandingan CRE daripada manusia dan haiwan makanan di Malaysia. Oleh yang demikian, kajian ini dijalankan dengan objektif umum untuk membandingkan genomik *Escherichia coli* (CREC) tahan karbapenem yang dipencilkan daripada manusia dan ayam. Kajian ini dijalankan ke atas strain arkib pencilan klinikal CREC persumtif (*n*=32) dari Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian dan 384 sampel swab kloaka ayam yang diperolehi dari Pantai Timur Malaysia (Kelantan, Terengganu, dan Pahang). Pengenalpastian bakteria dilakukan melalui kaedah rutin bakteriologi dan diikuti dengan pencirian fenotip dan molekul serta penentuan epidemiologi molekul menggunakan penaipan jujukan berbilang lokus (MLST). Penjujukan keseluruhan genom (WGS) Illumina HiSeqTM berdaya tinggi dilakukan terhadap sepuluh isolat CREC terpilih bagi menentukan genomik perbandingan bagi pencilan CREC tersebut. Anotasi genom isolat tersebut kemudiannya dilakukan menggunakan peralatan RASTtk, BAKTA, dan eggNOG-Mapper, dan pengukuran kuantitatif dan kualitatif untuk analisis hiliran ad-hoc dijana menggunakan pelayan M1CR0B1AL1Z3R (Microbializer). Analisis WGS dilakukan menggunakan ResFinder

4.1, VirulenceFinder 2.0, alat SerotypeFinder 2.0, FimTyper versi 1.0 CHTyper 1.0, cgMLST 1.2, pMLST (2.0), CSI Phylogeny, MobileElementFinder, ISFinder Hunter 1.7, ISFinder 1.7, ISFinder. Analisis genom komprehensif tambahan dilakukan menggunakan beberapa kaedah analisis genomik yang berbeza. Keputusan menunjukkan kadar pengesanan CREC secara keseluruhannya adalah sebanyak 7.29% (28/384) iaitu 10.94% (28) daripada 256 *E. coli* yang diasingkan daripada swab kloaka ayam, yang ditentukan melalui kaedah pengesanan fenotip. Daripada kesemua isolat CREC, didapati 40% (24/60) isolat adalah daripada manusia dan ternakan ayam yang mempunyai lebih daripada satu gen karbapenemase termasuk gabungan gen-gen bla_{NDM}+$bla_{\text{OXA-48}}$, bla_{NDM}+$bla_{\text{OXA-48}}$+bla_{IMP} dan $bla_{\text{OXA-48}}$+bla_{IMP}. Penaipan molekul menggunakan kaedah MLST menunjukkan pengesanan ST69, ST131, ST155, ST405, dan ST410 yang telah diiktiraf sebagai keturunan pandemik berisiko tinggi. Analisis genomik perbandingan menunjukkan persamaan rapat di antara isolat CREC daripada manusia dan ayam yang terbukti daripada hampir semua profil genomik termasuk filogeni, pulau genomik, analisis SNP, plasmid, serotyping dan cgMLST serta profil genomik dan proteomik lain. Hasil analisis genomik perbandingan menunjukkan persamaan di antara strain CREC daripada manusia dan ayam yang sihat dan ini merupakan data epidemiologi penting berkaitan CREC dalam manusia dan ayam di Malaysia. Penemuan daripada kajian ini dapat membantu dalam pemahaman epidemiologi CREC tempatan dan kemungkinan berlakunya penyebaran dinamik CRE dalam konteks tempatan. Hasil penemuan kajian ini seterusnya dapat membantu dalam merangka strategi kawalan dan pencegahan berasaskan bukti yang secara langsung dapat menyumbang kepada program kawalan kerintangan antimikrobial kebangsaan untuk menjaga kesihatan awam.

COMPARATIVE PHENOMICS AND GENOMICS OF CARBAPENEM-RESISTANT *Escherichia coli* FROM HUMANS AND BROILER CHICKENS

ABSTRACT

The emergence of carbapenem-resistant Enterobacterales (CRE) has been alarming, and its control has been considered one of the priorities set by the World Health Organization (WHO). In Malaysia, recent reports show that the prevalence of CRE in general and tertiary hospitals has been alarmingly rising. However, little is known about the occurrence of CRE in animals, particularly food-producing animals such as broiler chickens in Malaysia. Moreover, there is no study on the comparative study of CRE from humans and food animals in Malaysia. Therefore, this study was conducted with the general objective of elucidating the comparative genomics of carbapenem-resistant *Escherichia coli* (CREC) from humans and broiler chickens. The study was conducted on clinical isolates archives of presumptive CREC isolates (n=32) from Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, and 384 cloacal swab samples of broiler chickens collected from East Coast Malaysia (Kelantan, Terengganu, and Pahang). Routine bacteriology followed by phenotypic and molecular characterization and determination of molecular epidemiology using multilocus sequence typing (MLST) were conducted. High-throughput Illumina HiSeq™ whole genome sequencing (WGS) of ten selected CREC isolates was done to determine the comparative genomics of the CREC isolates. The assembled genomes were annotated using RASTtk, BAKTA, and eggNOG-Mapper tools, and quantitative and qualitative measurements for *ad-hoc* downstream analyses were generated using M1CR0B1AL1Z3R server (Microbializer). Analyses of the

WGS were done using ResFinder 4.1, VirulenceFinder 2.0, SerotypeFinder 2.0 tool, FimTyper version 1.0 CHTyper 1.0, cgMLST 1.2, pMLST (2.0), CSI Phylogeny, MobileElementFinder, Alien Hunter 1.7, ISFinder and IslandCompare (v1.0). Additional comprehensive genome analyses were done using different genomic analysis pipelines. The results showed an overall CREC detection rate of 7.29% (28/384) which is 10.94% (28) of the 256 *E. coli* isolated from cloacal swabs of broiler chickens based on phenotypic detection methods. Out of all the CREC, 40% (24/60) of the CREC isolates from human and broiler chickens harbor more than one carbapenemase gene, including the combinations bla_{NDM}+$bla_{\text{OXA-48}}$, bla_{NDM}+$bla_{\text{OXA-48}}$+bla_{IMP}, and $bla_{\text{OXA-48}}$+bla_{IMP}. The molecular typing using MLST showed the detection of ST69, ST131, ST155, ST405, and ST410, which have been recognized as high-risk pandemic lineages. The comparative genomic analyses showed close similarities between CREC isolates from human and broiler chickens, which were evident from almost all the genomic profiles, including phylogeny, genomic islands, SNP analysis, plasmid, serotyping, and cgMLST and other genomic and proteome profiles. The comparative genomic analysis results showing similarities among CREC isolates from humans and apparently healthy chickens are important epidemiological data on CREC in human and broiler chickens in Malaysia. The findings from this study can help in better understanding the local CREC epidemiology and shed light on the possible CRE transmission dynamics in the local context. These findings, in turn, can help in devising of evidence-based control and prevention strategies that can contribute to the national antimicrobial resistance control programs to safe guard the public health.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

The discovery of antimicrobials ushered in an era of hope that promised a medical revolution that enabled the treatment of several deadly diseases and immensely contributed to improved quality of life and life expectancy. Since the introduction of antibiotics on a large scale in the 1940s, deaths caused by infectious diseases have fallen by 70% (Placket, 2021). Over a few decades, several potent antibiotics were discovered and further improved the effectiveness of antimicrobial therapy by availing more options for treating formerly untreatable infectious diseases. However, this same era also witnessed the fast emergence and spreading of antimicrobial resistance among several pathogens. Antibiotic-resistant bacteria continue to increase in diversity and potency, and new species and strains of bacteria have been emerging and spreading worldwide. This problem is exacerbated by the fact that pharmaceutical companies appear to have come to terms with apparent 'surrender' to the ever-evolving nature of resistant pathogens rendering every effort to develop new antibiotics futile (Plackett, 2020; Carlet *et al*., 2014; WHO, 2014). The death toll due to infections caused by resistant bacteria is expected to rise from the current 700,000 to more than 10 million by 2050 (O'Neill, 2014). The annual economic impact of AMR was estimated to incur over US $105 billion in losses worldwide, and developing countries, particularly Africa, are projected to suffer much of the relative economic impact with a 20% reduction in the region's total economic output, which is equivalent to a reduction in GDP of US $2895 billion by 2050 (Codjoe and

Donkor, 2017). Infection with resistant pathogens leads to severe sickness, prolonged admission, increased healthcare and second-line drug costs, and treatment failures. For example, in Europe, the loss due to AMR-related costs has been estimated to be more than nine billion euros annually. In addition, the Centers for Disease Control and Prevention (CDC) estimated that AMR incurs an additional USD $ 20 billion in direct healthcare costs and USD $ 35 billion in indirect losses due to loss of productivity every year in the United States (Dadgostar *et al*., 2019).

The ever-increasing emergence and spread of AMR are attributed to several factors, including overpopulation, increase in global migration, imprudent and increased use of antibiotics in humans and animals, selection pressure of antibiotics on microorganisms, environmental changes, wildlife spread, poor sanitation, and lack of appropriate disposal of sewerage are among the significant contributors to the (Aslam *et al*., 2018). Among the several causes of AMR, the excessive use of antibiotics has been identified as a major driving factor in the evolution and spread of resistant bacteria. This has been demonstrated by epidemiological studies showing the correlation between antibiotic usage and the development of resistance in bacteria (Cock and Cuny, 2020; Read and Woods, 2014. In bacteria, resistance genes can be intrinsically presented or acquired from other bacteria through mobile genetic elements (MGEs) such as plasmids, insertion sequences, and transposons (Read and Woods, 2014). Apart from HGT, bacteria may also develop resistance spontaneously through mutation. The use of antibiotics creates selective pressure and removes drug-sensitive competitors favoring the survival of resistant bacteria due to natural selection (Read and Woods, 2014). Despite warnings regarding overuse, antibiotics are overprescribed worldwide (Ventola, 2015).

Antimicrobial resistance is a global threat to public health, animal health and production, food safety and food security, and environmental health. Multi-drug resistant (MDR) bacteria or "superbugs" continue to exist, emerge, and spread along the human-animal-environment interface with intertwined dynamics of sharing of resistance determinants among these triads. The common causes of AMR include overuse and misuse of antibiotics in humans and animals accompanied by poorly controlled antibiotics trading, increased international travel, poor sanitation and hygiene, and release of non-metabolized antibiotics and their residues into the environment through manure/feces. These factors facilitate the genetic selection pressure, which favors the emergence of infections caused by MDR bacteria in the community (van Boeckel *et al*., 2015). Antibiotics are also used in food-producing animals such as poultry, cattle, and pigs, and it is projected that an increase of up to 67% in such antibiotic uses will be recorded in highly populated countries (van Boeckel *et al*., 2015). Due to varying degrees of host specificities and the complexity of transmission, it is difficult to quantify the spread of resistant bacteria between humans and animals (Cock and Cuny, 2020; Muloi et *al*., 2018; Van Boeckel *et al*., 2015). However, initiatives taken to reduce antimicrobial usage in animals have shown some promise in reducing the occurrence of resistant bacteria in humans and animals (Stoica and Cox, 2021).

The global increase in demand for animal protein has become a worldwide phenomenon and dietary trend, notably in developing countries. Although meat production in high-income countries has plateaued since 2000, growth rates of 40%, 64%, and 68% were recorded in South America, Africa, and Asia, respectively. This growth and increased demand for animal protein in low- and middle-income countries (LMICs) have been enabled by the global expansion of improved animal production systems which

heavily rely on antimicrobials to maintain and enhance food animal health and production. Records show that up to 73% of all antimicrobials sold globally are used for food animal production. This scenario has been accompanied by a growing body of evidences showing the link between extensive antimicrobial use in food animal production and the rise of infections caused by antimicrobial-resistant pathogens both in animals and humans (Pokharel *et al*., 2020).

In recent years, ample evidence has shown that the public health challenges caused by AMR are increasing, and coordinated global interventions are required to contain these ever-increasing threats. The public health and economic burdens of AMR have been showing increasing trends, and worldwide data show that common and diverse bacterial pathogens have alarmingly become resistant to currently available antimicrobials (Codjoe and Donkor, 2018). To counter the rising threats of AMR, the World Health Organization (WHO) developed a ranking list of antimicrobial-resistant pathogens that can be used as a guide for determining areas of focus and effective resource allocation (WHO, 2017). Carbapenem resistance by Enterobacterales was identified as one of the pathogens that have been assigned a high critical priority.

Carbapenems are a group of antibiotics that have been used as a lifesaving and last-resort antibiotic to treat infections caused by MDR bacteria. Carbapenem-resistant Enterobacterales are among the major challenges to healthcare systems. Due to limited antimicrobials, infections caused by CRE are more challenging to treat and are commonly associated with high mortality and morbidity (Dong *et al*., 2020). Therefore, the ongoing increase of CRE prevalence in Enterobacterales species commonly associated with severe infections in healthcare settings is a matter of major concern. The development and spread of carbapenem resistance are mostly attributed to CRE and are mostly driven by the

emergence and spread of carbapenemases which are specific group carbapenem hydrolyzing beta-lactamases. Most carbapenemases-producing Gram-negative bacteria are resistant to carbapenems or are less susceptible to the same antimicrobials and other broad-spectrum agents (Iovleva *et al*., 2017).

In Malaysia, the carbapenem resistance rate in *E. coli* is still less than 1%, and a fluctuation in imipenem resistance was seen during the three-year periods (2018-2020) with prevalence rates of 0.8%, 0.5%, and 0.7% being recorded in 2018, 2019, and 2020 respectively. In comparison, the prevalence of meropenem-resistant *E. coli* increased from 0.6% in 2019 to 0.7% in 2020. Likewise, the prevalence of imipenem and meropenem-resistant *Klebsiella pneumoniae (K. pneumoniae*) also showed an increasing trend, with prevalence rates of 1.7% and 2.1% in 2019 to 2.4% and 2.8%, respectively, in 2020 (NSAR, 2020). According to ten-year (2006 to 2017) data compiled by the National Surveillance of Antimicrobial Resistance (NSAR) of Malaysia indicated that the overall prevalence of CREC declined from 0.5% in 2010 to 0.2% in 2014 (Hsu *et al*., 2017). However, a more recent report on the prevalence of CRE in a tertiary hospital in Malaysia shows that the prevalence of CRE in 2015 and 2016 was 0.3% (5/1590) and 1.2% (17/1402), respectively. The same study reported that the majority (81.8%) of the isolates were *Klebsiella pneumoniae*, followed by *Serratia marcescens*, *E. coli*, and *Citrobacter koseri* (Mohamed *et al*., 2018). However, the data on the prevalence of CREC in animals in Malaysia is scarce. Since the inception of this study, a single preliminary study by Ghazali *et al*. (2020) reported a CRCE prevalence of 1% (2/200) from antemortem cloacal swab samples collected from broiler chicken from an abattoir in Terengganu. However, this study was limited in scope and depth of investigation.

1.2 Justification of the study

Reports on the occurrence and prevalence of CRE in Malaysia showed that there is an alarmingly increasing trend in the incidences of CRE-related infections in general and tertiary hospitals in Malaysia (Zaidah *et al*. (2017). This may imply that the status of CRE in Malaysia is still not fully investigated, and the prevalence, diversity, resistance patterns, and different potential sources of CRE have not been sufficiently investigated. In addition, almost all the reported CRE prevalence studies in the country have been conducted in hospital settings, and the occurrence and characteristics of CREC from animals, particularly CREC from food animals, have not been well investigated. There is also a paucity of data on the occurrence of CREC and whether broiler chickens may serve as CREC that may spread to humans and possibly cause infections in Malaysia. In addition, there is no data on the comparative genomics of CREC isolates from humans and broiler chickens in Malaysia. Understanding the antimicrobial resistance patterns, virulence profiles, molecular epidemiology, and genomic characteristics of CREC from human and broiler chickens will provide a detailed and better insight into the existing status of these pathogens and may help complement the national AMR control strategy.

1.3 Research questions

1. How prevalent is CREC in broiler chickens in East coast Malaysia (Kelantan, Terengganu, and Pahang)?
2. What are the antimicrobial resistance, virulence, and phylogenetic characteristic of CREC isolates from humans and broiler chickens?

3. What is the molecular epidemiology of CREC isolates from human and broiler chickens, and how the local CREC strains are related to the globally disseminated *E. coli* strains?
4. What is the comparative genomics of CREC isolated from humans, and how does this comparative study help in generating epidemiologically useful insights?

1.4 Objectives of the study

1.4.1 General objectives

1. This study aimed to investigate the comparative genomics of carbapenem-resistant *E. coli* (CREC) from humans and broiler chickens in East Coast Malaysia (Kelantan, Terengganu, and Pahang)

1.4.2 Specific objectives

2. To determine the prevalence of CREC in broiler chickens in East coast Malaysia (Kelantan, Terengganu, and Pahang)
3. To determine antimicrobial resistance patterns of carbapenem-resistant *E. coli* isolates from broiler chickens and humans.
4. To conduct molecular characterization of CREC isolates based on the detection of carbapenemases genes, virulence genes, and phylogenetic characteristics.
5. To determine the molecular epidemiology of carbapenem-resistant *E. coli* isolates
6. To elucidate the comparative genomics of carbapenem-resistant *E. coli* isolates from human and broiler chickens.

CHAPTER TWO

LITERATURE REVIEW

2.1 General characteristics of *Escherichia coli*

Escherichia coli is a member of the *Enterobacterales* and is characterized as a short, non-spore-forming, facultatively anaerobic Gram-negative bacillus. It readily grows on ordinary media without the need for special enrichment. Biochemically, it is characterized by indole production, absence of citrate fermentation, positive reaction on methyl red test, and negative Voges–Proskauer reaction with no urease production (Bhutia *et al*., 2021). *Escherichia coli* is a predominant aerobic commensal and a member of gut microbiome of vertebrates. The majority of *E. coli* strains are commensals of the intestinal tract of warm-blooded animals and humans. In general, commensal *E. coli* are harmless and symbiotically live within the host while causing infections rarely in immune-competent hosts (Ramos *et al*., 2020). The bacteria is present in almost 90% of humans and is commonly found at a concentration of 107 to 109 colony- forming units (CFU) per gram of feces. *Escherichia coli* is also an opportunistic pathogen with high pathogenic potential to cause intestinal and extraintestinal infections (Denamur *et al*., 2021).

Pathogenic and non-pathogenic strains of E. *coli* are differentiated depending on their acquisition of virulence factors or loss of functional genes encoding adhesion, invasion, colonization, cell surface molecules, secretions, transport, survival, and iron metabolism (Sora *et al*., 2021). In terms of their genomes, *E. coli* strains may have varying numbers of genes ranging from 4,000 to 5,000. Among these, about 3,000 genes are present in different *E. coli* strains, while the rest of the genes are mostly associated with the genes that are responsible for colonization or virulence. The use of advanced genomic

tools such as next-generation sequencing (NGS) has enabled a clearer understanding of the plasticity of *E. coli* genomes by revealing much of the core and accessory genomes of both commensal and pathogenic *E. coli* strains (Poirel *et al*., 2018).

2.2 Pathogenic *Escherichia coli*

Escherichia coli strains cause several extraintestinal pathologies, including various intra-abdominal, pulmonary, soft tissue, skin, and urinary tract infections, new born meningitis (NBM), and bacteremia. The major intestinal infections caused by *E. coli* include different forms of diarrhea, including hemolytic and uraemic syndrome (HUS). Infections such as urinary tract infections (UTIs), renal failure in HUS in children, and neurologic complications in NBM are often associated with high mortality and morbidity.

In recent years, the incidences of extraintestinal infections caused by *E. coli* have been increasing, and HUS epidemics, such as the 2011 epidemic in Europe, have become more common. This problem has been further aggravated by the rising incidence and spread of antibiotic-resistant *E. coli*, making this pathogen the third-ranked 'priority pathogen' among the 12 antibiotic-resistant pathogens listed by the WHO (Denamur *et al*., 2021). In animals, *E. coli* is one of the major causes of diarrhea, along with other pathogens such as rotavirus, coronavirus, *Cryptosporidium parvum*, or a combination of these pathogens (Poirel *et al*., 2018). The bacterium can also cause UTIs in small animals (Teh, 2022).

In chickens, *E. coli* infections cause several disease syndromes, including septicemia, enteritis, omphalitis, respiratory tract infection, swollen head, and cellulitis (Swelum *et al*., 2021). Pathogenic *E. coli* strains cause various diseases through multiple

mechanisms of pathogenesis, including the colonization of the mucosae, host immune evasion, replication, and tissue injury.

Pathogenic *E. coli* are classified into pathotypes based on pathogenicity mechanisms (patterns of attachment and invasion), virulence (toxin production, presence or absence of virulence plasmids, mechanisms of attachment), and the clinical syndromes they cause (Kaper *et al*. 2004). These pathotypes are enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), including Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Pakbin *et al*., 2021). The different *E. coli* pathotypes and their characteristics are summarised in table 2.1.

2.3 Antibiotics Discovery and the ensuing medical Revolution

Antibiotics are chemical substances that are secreted by microorganisms or synthetic products with bacteriostatic or bactericidal properties and are used to inhibit the growth of or kill pathogenic bacteria (Bhattarai *et al*., 2020; Pancu *et al*., 2021). Throughout the ages, infectious diseases have been challenging human existence and quality of life. One of the marked historical pieces of evidence of the cataclysmic effects of infectious diseases was the emergence and spread of the Bubonic plague, which claimed the lives of approximately one-third of Europe's population between 1347 and 1350. In general, infectious diseases remained the leading causes of death up to the early 1900 (Ribeiro da Cunha *et al*., 2019).

Table 2.1. *Escherichia coli* pathotypes and their main characteristics.

Pathotype	**Identification criteria**	**Affected host (s)**	**Common Diseases and Symptoms**	**Major virulence genes**	**Phylog roups**	**Common sequence types (STs)**	**References**
EPEC	Adheres to the intestinal epithelium and effaces microvilli.	Humans, Domestic animals, chicken	Diarrhea in children, Watery diarrhea and vomiting	Bfp, Intimin, LEE	B2, C, D, F	ST131, ST88, ST69, ST62	Denamur *et al.* (2020) Kaper *et al*. (2004)
EHEC/ STEC	Shiga toxin production (Presence of *stx* genes)	Human, Cattle, Sheep	Hemorrhagic colitis, HUS, Bloody diarrhea	*stx*, *eae*, *ehxA*	B1, E	ST11 ST29 ST17	Garcia and Fox (2021)
ETEC	Production of adhesins enterotoxins	Human Pig Cattle Sheep	Traveler's diarrhea, Watery diarrhea and vomiting	LT, STa, STb, EasT, F4	A, B1, C, E	Multiple	Dubreuil *et al.* (2016)
EAEC	Aggregative adhesion on enterocytes	Human, domestic mammals	Diarrhea in children, Diarrhea with mucus and vomiting	Aggregative adherence fimbriae (*aaf/agg*) and	aatA	Multiple	Riley (2020)
EIEC	Colonocyte invasion	Human	Shigellosis-like, Watery diarrhea; dysentery	*ipa*C, *ipa*H, isc, var, Shiga toxin, hemolysin, Cellular invasion, Ipa	A, B1, E	ST6 ST270 ST280	Denamur *et al.* (2020), Garcia and Fox (2021)
DAEC	Diffuse adhesion on enterocytes	Human	Acute diarrhea in children, Watery diarrhea, recurring UTI,	Adhesion encoding genes (*afa* & *dra*), Daa, AIDA	All phylog roups	Multiple	
ExPEC	Extra-intestinal infection	Human, domestic mammals, poultry	Various extra-intestinal infections	Adhesion encoding genes, toxins,protect ins &iron capture systems	B2, C, D, F	ST131, ST69, ST88, ST62	Allocati *et al* (2013), Denamur *et al.* (2020),
ExPEC (APEC)	Isolated from birds	Poultry Human	Collibacillosis	pColV genes, Type 1 and P fimbriae; K1 capsule	B2, C	ST95, ST88	Denamur *et al.* (2020), Allocati *et al*. (2013)

The discovery and use of antibiotics radically impacted human health and quality of life to the extent that it was considered a 'medical miracle' of the 20th century. The years 1930–1962, which is often called the golden age of antimicrobial discoveries, saw the rapid invention and development of 20 different classes of novel antimicrobials, many

of which contributed to the betterment of human health and wellbeing for more than six decades (Dhingra *et al*., 2020). The 'miraculous' effects of antibiotics were later tapped for non-therapeutic applications, including their use in enhancing agricultural productivity, particularly as growth promoters to increase food production (Manyi-Loh *et al*., 2018).

The golden age of antibiotics was marked by overwhelming success in treating many life-threatening infectious diseases. This resulted in the overly optimistic view that diseases caused by microorganisms would finally be conquered in a very short period owing to the rapid antibiotics discoveries which were believed to enable the control of infectious diseases as a public health problem (Aminov, 2010). A notable remark of triumph on infectious diseases with the help of the apparently indomitable antibiotics was made by the US Surgeon General in 1970, who was said to opine that 'it was time to close the book' on infectious diseases and redirect national resources to the control and prevention of chronic problems such as cancer and heart disease (WHO, 2018). However, that was not meant to materialize as all the euphoric optimisms about antibiotics 'miracles' soon began to be shredded as resistant bacteria emerged. Even then, apparently, no one could have fully grasped the microbial resolve and capabilities enabling the pathogens to attain the status of apparent invincibility attributed to their ability to resist multiple antimicrobials, including resistance to the most potent antibiotics.

2.4 Mechanisms of Action of Antibiotics

At therapeutic concentrations, antibiotics are sufficiently potent to be effective against infection while simultaneously presenting minimal toxicity to the patient. Based on their action on bacteria, they are categorized as bactericidal or bacteriostatic. Natural

antibiotics originate from different species of bacteria and fungi as secondary metabolic products. As these substances are not essential for bacterial cell survival, they are usually produced in demand. Usually, bacteria produce these antibiotics against other competing bacteria and persist in challenging environmental conditions. In general, antibiotics that occur in their natural forms are less potent and have fewer side effects compared to synthetic antibiotics. Common natural antibiotics include penicillin, streptomycin, gramicidin, and chlortetracycline. Synthetic antibiotics are produced in the laboratory and approved for clinical use. Common examples of synthetic antibiotics include cephalosporin C, fluorocyclines, linezolid, and meropenem. Compared to natural antibiotics, synthetic antibiotics act faster and have higher toxicity to pathogens (Upmanyu and Malviya, 2020). The selective toxicity of these substances induces bactericidal and bacteriostatic to the bacterial cells with minimum side effects to the patients. The selective blocking of critical bacterial metabolic pathways disrupts bacterial cell structures (Abushaheen *et al*., 2020; Walsh, 2004). The different mechanisms of actions of groups of antimicrobial agents by which they kill or inhibit bacteria are discussed in the following sections.

2.4.1 Interfering with cell wall synthesis

The cell wall of a bacterial is the outermost elastic structure that maintains the bacterial cell structural integrity by protecting the bacteria from adverse osmotic effects that may cause bacterial cell disintegration. The peptidoglycan layer is made of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) long glycan chains cross-linked by Penicillin Binding Proteins (PBP). This bacterial structure is the main target for β-lactam and glycopeptides antibiotics (Nikolaidis *et al*., 2014). By acting on

the peptidoglycan layer of the bacterial cell wall, these antibiotics bacterial cell wall synthesis and disrupt the cell wall structure, thereby inducing bacterial cell lysis. Such antibiotics include glycopeptides (vancomycin and teicoplanin) and *β*-lactams (carbapenems, penicillins, cephalosporins, and monobactams).

The β-lactam antibiotics are a wide class of antibiotics produced by the fungus *Penicillium* and were discovered in the 1930s. These antibiotics are characterized by the presence of an azetidinone nucleus containing the carbonyl β-lactam, which is essential for the activity (Kapoor *et al*., 2017). There are several classes of *β*-lactam antibiotics that target the penicillin-binding proteins (PBPs) and are used against different bacterial species. The *β*-lactam antibiotics are structurally similar to the D-Ala-D-Ala dipeptide of the developing peptidoglycan to which it covalently binds at the serine active binding site on the PBPs blocking the formation of linkage between the peptidoglycan layer, which ultimately blocks cell wall synthesis (Zapun *et al*., 2008). The *β*-lactams are the most popular bactericidal antibiotics the most common and popular antibiotics used to treat several bacterial infections and usually have lower toxicity with the exception of allergic reactions in sensitive individuals (Balsalobre *et al*., 2020).

2.4.2 Inhibition of protein synthesis

Protein synthesis is a complex and essential biological process in living cells that occurs through processes including transcription and translation, which are carried out through initiation, elongation, termination, and recycling. The differences in structures of the bacterial ribosome and the ribosome of eukaryotes enable the selective inhibition of bacterial protein synthesis. The antibiotics achieve the inhibition by blocking the protein synthesis process at the 30S or 50S subunits of the 70S bacterial ribosome. By inhibiting

protein synthesis, the antibiotics stop or retard bacterial cell growth (Tenover, 2006). Common examples of 30S subunit-blocking antibiotics are Macrolides, aminoglycosides, and tetracycline. The positively charged carbohydrate groups of the antibiotics bind to the negatively-charged plasma membrane and diffuse into the bacterial cell. Once inside the bacteria cell, they attach to the 30S subunit of the ribosome at the A-site, reversing the process into extra-helical translation, which causes the formation of a faulty mRNA-tRNA pairing that leads to errors in translation and protein synthesis (Wilson, 2014; Garneau-Tsodikova and Labby, 2016). Whereas the 50S ribosome subunit of the bacterial cell forms a polypeptide chain in the peptidyl transferase center (PTC). Moreover, the 50S subunit contains a nascent peptide exit tunnel (NPET) which serves as a gate to the polypeptide chain leaving the ribosome. A common example of 50S subunit inhibiting antibiotics is chloramphenicol which acts by binding to the 50S subunit between the NPET and the PTC, which prevents the incorporation of newly made polypeptides cross the channel. This leads to disruption in the elongation step and results in the inhibition of bacterial protein synthesis (Ban *et al.*, 2000).

2.4.3 Inhibition of nucleic acid synthesis

The synthesis of bacterial DNA requires topoisomerases; the lack of these enzymes leads to the formation of abnormal DNA (Abushaheen *et al*., 2020; Pommier *et al*., 2010). For example, fluoroquinolones function by inhibiting the enzyme DNA gyrase enzyme in Gram-negative bacteria, which is vital in initiating bacterial DNA replication. They also inhibit the enzyme topoisomerase IV, which is critical for the daughter-cell segregation in Gram-positive bacteria. The quinolones bind to topoisomerase IV or II and impede bacterial DNA synthesis by modifying the supercoiling of DNA, causing the

interruption of double-stranded bacterial DNA and leading to the death of the bacteria. The antibiotic effect is achieved through the pathway that may or may not depend on protein synthesis pathways (Abushaheen *et al*., 2020).

2.4.4 Inhibition of metabolic pathways/bacterial enzymes

The metabolic processes and synthesis of various cellular components of prokaryotic and eukaryotic cells require reduced folate co-factors as a vital component. In eukaryotic cells, the uptake of folate occurs through an active transport system, whereas in prokaryotic cells acquire folate through the de novo synthesis pathway. This makes the folate biosynthesis pathway a viable target for antibiotics (Bertacine Dias *et al*., 2018). The folate synthesis pathway utilizes the enzyme dihydropteroate synthase (DHPS), which requires para-aminobenzoic acid (PABA). Sulphonamides act by inhibiting PABA in bacterial folate synthesis. The fact that sulphonamides share structural similarity with PABA makes it a competitive inhibitor to which folates can bind as alternatives which in turn deprives the bacteria cell of the vital nutrient, thereby leading to inhibition of the bacterial cell growth. Diaminopyrimidine antibiotics (i.e., Trimethoprim) inhibit dihydrofolate reductase (DHFR), which is the last enzyme in the folate biosynthesis pathway (Schober *et al*., 2019). By incorporating it into the precursors, sulfonamides block the formation of folic acid and form a reactive and antibacterial pseudometabolite. Sulfonamides are bacteriostatic antibiotics with antifungal and antimalarial properties. The combination of sulphonamide and diaminopyrimidine antibiotics has been used in the treatment of several infectious diseases, including urinary tract infections. However, the emergence of remittance against these antibiotics combination proved to be a challenge (Giles *et al*., 2019). Despite their common side effects, sulfonamides are considered to be

among the most effective and safe antibiotics in WHO's list of essential medicine (WHO, 2015).

2.4.5 Interruption of bacterial membrane

The bacterial membrane plays a vital role in ensuring bacterial cell survival and thus can be a good target for antimicrobial agents. Unlike Gram-positive bacteria, Gram-negative bacteria have an added protective layer on the outer membrane composed of lipopolysaccharides (LPS). Several potent antimicrobial agents hinder the formation of mature LPS by inhibiting LPS synthesis at various stages. As a result, the bacteria become more prone to imbalances in osmotic pressure due to the increasing permeability that leads to bacterial cell destruction (Epand *et al*., 2016). Polymyxins which are considered the last-line antibiotics for the treatment of infections caused by MDR Gram-negative bacteria, are common examples of bacterial cell membrane synthesis inhibiting antibiotics. The bacteriostatic action of Polymyxins is achieved through the interaction between polymyxin, a positive charge, with the lipid A of LPS, which is negatively charged. This binding alters the bacterial structure and makes the cell membrane extra permeable. This, in turn, leads to disruptions in osmotic pressures in the bacterial cell leading to the outflow of cellular components, inhibition of respiration, and surge of water inflow, which finally leads to lysis and death of the bacterial cell (Yin *et al*., 2020). Figure 2.1 shows the summary of the major mechanisms of antimicrobial actions and their corresponding potential mechanisms of resistance.

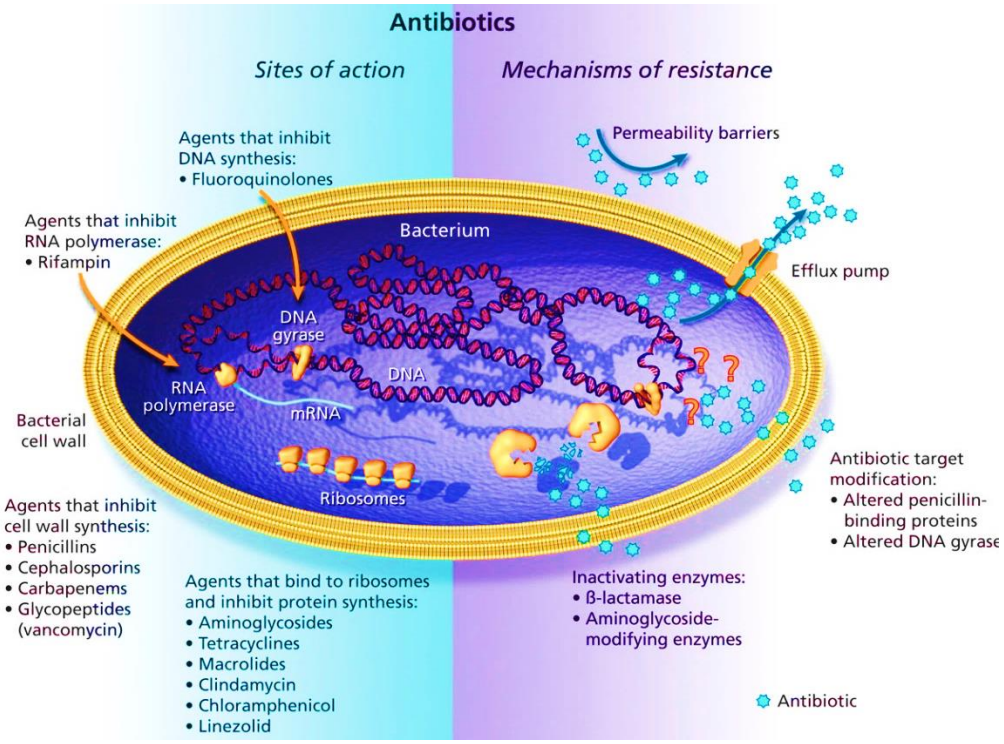

Figure 2.1. Sites of action and potential mechanisms of bacterial resistance to antimicrobial agents. Adapted from (Mulvey and Simor, 2009)

2.5 Antimicrobial Resistance

Antimicrobial Resistance occurs when pathogens such as bacteria, viruses, fungi, and parasites evolve through time and no longer respond to antimicrobial therapy, thereby making it increasingly difficult or impossible to treat infections and increasing the severity of illness, transmission, and death (WHO, 2021). Antimicrobial resistance has been an acknowledged fact since the dawn of the antibiotic era. In fact, it did not take long for resistant bacteria to emerge soon after the discovery of antibiotics and their introduction into clinical use. However, the threats of AMR became of serious concern only in recent decades following the emergence of diverse and dangerous resistant strains, which continues to occur at an alarming rate. This escalating evolution of resistance by bacteria

coupled with an apparently exhausted and lessened antibiotic pipeline has somehow caused the fear that the ushering of a post-antibiotic era is apparently eminent (Fair and Tor, 2014, Jackson *et al*., 2018). Antibiotic resistance is a global public health threat that affects individuals, communities, societies, and countries all over the world. The impacts of AMR are not only limited to health care but also to veterinary and agricultural sectors. Resistant bacteria have been evolving and increasingly posing enhanced resistance levels characterized by higher frequency and strength of resistance against all antibiotics that have been approved for clinical use worldwide. This has led to the shortening of the clinical usability life span of newly approved antibiotics to less than ten years before high incidences of resistance demand the guarded usage of these antibiotics (Spagnolo *et al*., 2021). The emergence of AMR is a multifaceted and complex issue that has been compounded by several contributing factors. Although the common notion assumes that AMR emerged as a result of the introduction of antibiotic usage in health care and other sectors, evidence suggests otherwise and that resistant bacteria existed well before the discovery and clinical use of antibiotics. This can be illustrated by the fact that penicillin-resistant Staphylococcus species were identified even before the discovery, industrial production, and widespread clinical usage of the first antibiotic in 1943 (Hwang and Gums, 2016). This observation is explained by the fact that the genetic diversity required for the development of penicillin resistance in Staphylococcus could not have developed in the shorter time frame following the introduction of penicillin in clinical use. Rather it implies that bacteria do have an intrinsic resistance encoded in their genome that has been evolving over centuries. Thus, the development of antimicrobial resistance in bacteria is a natural process that occurs with or without human intervention (Fair and Tor, 2014). However, the introduction of penicillin in clinical use was believed to create selective

pressure on bacteria which induced bacterial adaptive mechanisms that enabled the acceleration of natural selection and the emergence of more resistant or more virulent bacteria. This problem was further aggravated by the widespread introduction of multiple potent antimicrobials and their imprudent use in humans, animals, and agriculture (Hwang and Gums, 2016; Fair and Tor, 2014).

2.5.1 Mechanisms of antimicrobial resistance

As part of their evolution processes that took place over millions of years, diverse species of bacteria developed complex and sophisticated mechanisms of survival in the presence of antimicrobial molecules. Such an interesting sophistication in bacterial cell resistance is their ability to resist a particular class of antibiotics through multiple biochemical pathways, which helps the bacteria to have a tool kit of mechanisms to evade the effect of antibiotics (Munita and Arias, 2016. In general, AMR mechanisms are broadly classified into intrinsic (natural) and acquired resistance. Intrinsic or natural resistance refers to the inherent nature of bacterial species to be resistant to some antibiotics due to their unique structural/functional characteristics. Whereas acquired resistance is the development of antibiotic resistance by naturally susceptible bacteria through the acquisition of specific genetic codes from other bacteria (Abushaheen *et al*., 2020).

2.5.1(a) Genetic Basis of Antimicrobial Resistance

Endowed with higher genetic plasticity, bacteria are capable of responding to arrays of threats from their environments, including the presence of antimicrobial

molecules that may threaten their existence. Because they share the same ecological niches with antimicrobial-producing microorganisms, different species of bacteria have evolved primeval mechanisms to thwart the potentially deadly effects of antibiotic molecules to ensure their survival. The two major genetic strategies used by bacteria that are of evolutionary importance to survive and thrive in the presence of antimicrobial substances are through mutations in gene(s) that are often associated with the mechanism by which the antimicrobial compounds act on the bacteria and through the acquisition of AMR encoding foreign DNA through HGT (Munita and Arias, 2016; Peterson and Kaur, 2018).

2.5.1(b) Mutational Resistance

Because of the diverse and intricate mechanisms of mutation, antibiotic resistance acquired through mutational changes are diverse and complex. In general, bacterial cell mutation resulting in the development of antimicrobial resistance interferes with the action of antibiotics through one of the following mechanisms, *i)* antimicrobial target modifications, *i)* reduced drug uptake, *ii*) activation of efflux mechanisms, *iv)* global alteration of vital metabolic pathways through modulation of regulatory networks (Lopatkin *et al*., 2021; Munita and Arias, 2016). Specific examples of antimicrobial resistance development through mutational changes will be discussed in the following sections.

2.5.1(c) Horizontal Gene Transfer

Acquisition and incorporation of foreign DNA material from other bacteria or environments through HGT are vital driving factors that enormously contribute to the

evolution of bacterial pathogens and their ability to develop AMR (Munita and Arias, 2016). Most of the antimicrobials in clinical use are derived from natural environmental products, including soil. As stated earlier, bacteria living in environments with natural antimicrobials harbor intrinsic genetic determinants of resistance, and there is ample evidence suggesting that environmental resistome is a robust source of antimicrobial resistance acquisition by pathogenic bacteria. The genetic exchange of resistance genes has been frequently implicated as the cause of AMR emergence and dissemination. Naturally, three main strategies are used by bacteria to acquire external genetic material, i) conjugation (bacterial "sex"), ***ii***) transduction (phage mediated), and transformation iii) (incorporation of naked DNA).

Transformation is considered the simplest type of HGT; however, under natural circumstances, only very few bacteria with clinical relevance are able to incorporate naked DNA and develop resistance. Nosocomial emergence of resistance is often attributed to conjugation, which is a more efficient gene transfer method through cell-to-cell contact between the donor and recipient bacteria. It has been shown that conjugation is more likely to occur at high rates in the gastrointestinal tract of humans receiving antibiotic treatment (Niel *et al*., 2021). Although the direct transfer of resistance genes from chromosome to chromosome is possible, conjugation mostly occurs through the movement of MGEs. Among the MGEs, plasmids and transposons play important roles in the emergence and dissemination of AMR among clinically important bacteria (Munita and Arias, 2016; Partridge *et al*., 2018). Lastly, integrons are ancient structures that promote bacterial evolution through the acquisition, storing, disposing, and resorting of reading frames in mobile gene cassettes. They are considered one of the most efficient mechanisms for accumulating AMR genes. Integrons are one of the main drivers of AMR as they provide

an efficient yet simple mechanism for the incorporation of new genes into bacterial chromosomes, maintenance of the functional equipment, and a robust strategy of the genetic interchange (Sabbagh *et al*., 2021).

2.5.1(d) Mechanistic Bases of Antimicrobial Resistance

Based on the biochemical route involved in resistance, antibiotic resistance mechanisms can be categorized as follows: (i) drug uptake limitation, (ii) drug target modification, (iii) drug inactivation; and (iv) drug efflux (figure 2.2). Because of their structural differences and others, all four mechanisms are used by Gram-negative whereas since Gram-positive bacteria lack the lipopolysaccharide in the outer membrane, they are less likely to use limiting the uptake of a drug and drug efflux mechanisms (Reygaert, 2018; Uddin *et al*., 2021). Each of these resistance mechanisms has its own specific biochemical pathways that will be further elaborated in the following section.

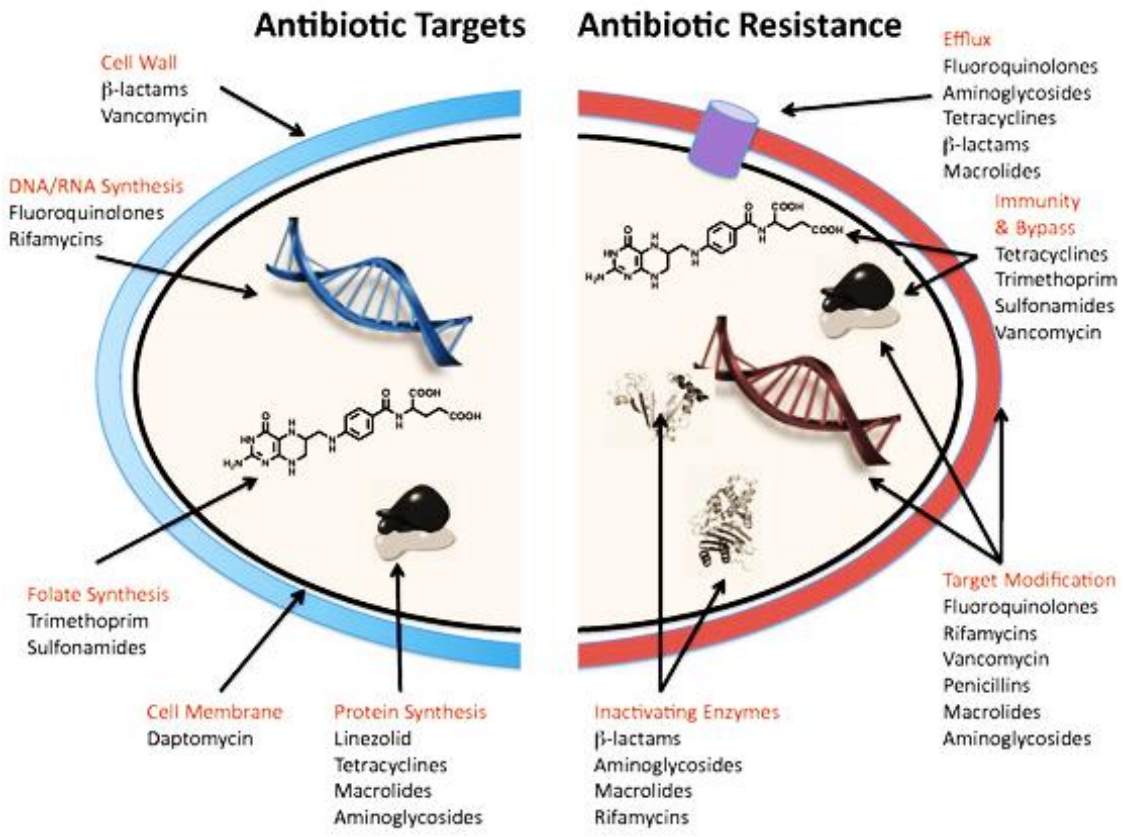

Figure 2.2. Antibiotic targets and mechanisms of resistance. Adapted from (Wright (2010)

2.5.1(e) Drug uptake limitation

Naturally, bacterial species differ in their abilities to limit the uptake of antimicrobial agents. For example, the structural and functional features of the Lipopolysaccharides (LPS) layer in Gram-negative bacteria serves as a barrier to certain types of molecules, arming the bacteria with intrinsic resistance to several groups of antibiotics (Bertani and Ruiz, 2018). It is due to this structural difference that glycopeptide antibiotics such as vancomycin are incapable of penetrating the outer membrane barrier and are, therefore, not effective against Gram-negative bacteria. The net change in permeability across the cell membrane barrier is determined by the hydrophobicity of the antibiotics. Hydrophobic antibiotics diffuse through the membrane, while hydrophilic